SENSITIZATION OF BREAST CANCERS TO HYPOXIA- OR DEATH RECEPTOR-INDUCED APOPTOSIS BY INHIBITION OF NF-KAPPA B

Rajani Ravi and Atul Bedi

Johns Hopkins University

rbedi@jhmi.edu

The NF-kB (Rel) family of heterodimeric transcription factors plays an important role in determining cell survival during immune, inflammatory, and stress responses. NF-kB dimers containing RelA or c-Rel are held in an inactive cytoplasmic complex with inhibitory proteins, the IkBs. Phosphorylation of IkBs by the multi-subunit IkB kinase complex (IKK: IKKalpha, IKKbeta, and IKKgamma/NEMO) targets them for rapid ubiquitin-mediated degradation. In addition to the release and nuclear translocation of the dimer, transcriptional induction of target genes by NF-kB requires phosphorylation of Rel proteins by Casein Kinase II (CK2).

We find that the frequent activation of IKK and CK2 by diverse genetic aberrations and growth factor receptors (HER2/neu or IGF-1R) results in the constitutive activation of NF-kB in breast cancers. NF-kB promotes the expression of a number of anti-apoptotic genes, including c-FLIP, members of the Inhibitor of Apoptosis family, and the Bcl-2 homolog, Bcl-xL. We find that breast cancer cells exposed to hypoxia (0.1% O2) remain viable and exhibit hypoxia-inducible factor-1 (HIF-1)-mediated transcriptional activation of the vascular endothelial growth factor (VEGF) gene. However, repression of NF-kB-dependent survival gene expression with either IKK inhibitors (NEMO binding peptides or acetyl salicylic acid) or CK2 inhibitors (Apigenin, Emodin, or DRB) sensitizes breast cancer cells to hypoxia-induced apoptosis. While hypoxia-induced p53 can trigger an 'intrinsic' pathway of cell death via mitochondrial activation of caspase-9, it also promotes expression of death receptors for Apo2L/TRAIL (TNF-related apoptosis inducing ligand). We find that breast cancer cells are protected from Apo2L/TRAIL-induced apoptosis by NF-kB-induced expression of proteins that inhibit the caspase-8-BID-BAX death signaling pathway. Conversely, breast cancer cells can be sensitized to Apo2L/TRAIL-induced death by inhibition of IKK and CK2.

Our results indicate that NF-kB is a critical determinant of the resistance of breast cancers to hypoxia- or death receptor-induced apoptosis and suggest that the combination of Apo2L/TRAIL with IKK/ CK2-inhibitors may be a highly effective regimen for treatment of breast cancers.

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HEAT SHOCK PROTEIN 27 INHIBITS APOPTOSIS IN HUMAN BREAST CANCER CELLS BY BINDING TO CYTOCHROME C

Stephen W. Carper

Chemistry Department and University of Nevada,
Las Vegas, Cancer Institute, University of Nevada,
Las Vegas, NV

carpers@unlv.edu

Several studies have shown that elevated levels of heat shock protein 27 (hsp27) in breast cancer results in poor prognosis for the patient. Tumors recur at much high frequencies if the levels of hsp27 are elevated. We have genetically engineered human breast cancer cells to constitutively express hsp27. These cell lines are better able to survive cytotoxic stresses that induce apoptosis. To investigate the molecular mechanism of hsp27 inhibition of apoptosis we measured caspase activity in the transfected cell lines. Hsp27 expressing cells fail to show activation of caspase 3 or caspase 9 following exposure to sodium butyrate, an inducer of apoptosis. In vitro studies using recombinant caspase 3, caspase 8, caspase 9 and hsp27 indicated that hsp27 does not directly inhibit caspase activity. Cytochrome c is released from mitochondria when cells undergo apoptosis. Cytochrome c forms a complex with dATP, Apaf-1 and procaspase 9 which results in the activation of caspase 9 and ultimately the activation of caspase 3. Since hsp27 does not directly inhibit caspases yet can inhibit their activation we decided to measure the ability of hsp27 to interact with cytochrome c. Immunoprecipitation experiments show that hsp27 binds to cytochrome c which can prevent it from activating caspase 9 and hence caspase 3. Granzyme b, a serine protease which directly activates caspases, was able to activate caspase 3 in the presence of hsp27. These results indicate that hsp27 is a novel inhibitor of apoptosis. They also indicate that potentially treating breast cancers which express hsp27 with chemotherapeutic agents would not be as successful as using an immunotherapy approach.

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THE ROLE OF C-MYC OVEREXPRESSION IN SENSITIZATION OF MAMMARY EPITHELIAL CELLS TO APOPTOSIS

Christine M. Coticchia and Robert B. Dickson

Lombardi Cancer Center,
Georgetown University Medical Center

coticchc@georgetown.edu

The c-myc oncogene is thought to play an important role(s) in the onset and progression of breast cancer, where it is amplified in approximately 15% and overexpressed in over 60% of human breast cancers. Depending upon the availability of survival factors, cells that constitutively express c-Myc undergo proliferation, growth arrest, or apoptosis through as yet poorly defined mechanisms. Mammary tumors from c-myc transgenic mice are characterized by a long tumor latency and an elevated apoptotic index. Currently the molecular mechanism by which c-Myc sensitizes cells to apoptosis is not fully understood. Also unclear is the exact type of stimuli to which c-Myc-overexpressing cells respond to apoptotically, and what environmental conditions in c-Myc-overexpressing mammary epithelium promote apoptosis versus proliferation and transformation. Currently, the molecular mechanisms by which c-Myc has been shown to mediate apoptosis are the Fas/Fas ligand pathway, p53 and mitochondrial-dependent release of cytochrome C. Previously it has been demonstrated in our laboratory that a mammary carcinoma cell line (Myc83), derived from MMTV-c-myc transgenic mouse, are sensitive to EGF withdrawal and undergo extensive apoptosis upon inhibition of EGFR signaling. Apoptosis of Myc83 under these conditions was accompanied by the up-regulation of the death receptor Fas, but not of its ligand FasL, as previously thought. This apoptosis was preceded by the activation of the pro-apoptotic stress related MAPK, p38.

The present project studied c-Myc's sensitization to apoptosis of both Myc83 cells, and a non-tumorgenic immortalized mouse mammary epithelial line overexpressing c-Myc (FSK7-Myc) compared to the control FSK7-LXSN cells. Here we show that growth factor withdrawal/blockade, rFasL, and other apoptotic stimuli resulted in apoptosis in Myc83 and FSK7-Myc cells but not in the control FSK7-LXSN cells, suggesting that c-Myc overexpression in mammary epithelial sensitizes them to apoptotic stimuli. However, evaluation of the mechanism responsible for apoptosis induced by growth factor withdrawal in Myc-expressing cells revealed a lack of a role for both endogenous Fas/FasL and p38-MAPK in mediating this c-Myc sensitization to apoptosis.

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PHOSPHORYLATION ON TYROSINE-15 OF P34CDC2 BY ERBB2 INHIBITS P34CDC2 ACTIVATION AND IS INVOLVED IN RESISTANCE TO TAXOL-INDUCED APOPTOSIS

Ming Tan, Tong Jing, Keng-Hsueh Lan, Christopher L. Neal, Ping Li, and Dihua Yu

University of Texas M.D. Anderson Cancer Center,
Department of Surgical Oncology,
1515 Holcombe Boulevard, Houston, TX 77030

dyu@notes.mdacc.tmc.edu

ErbB2 overexpression confers resistance to taxol-induced apoptosis by inhibiting p34Cdc2 activation. One mechanism is via ErbB2-mediated upregulation of p21Cip1, which inhibits Cdc2. Here, we report that the inhibitory phosphorylation on Cdc2 tyrosine (Y)15 (Cdc2-Y15-p) is elevated in ErbB2-overexpressing breast cancer cells independent of Wee1, Cdc25C, and p21Cip1. ErbB2 binds to and colocalizes with cyclin B-Cdc2 complexes. ErbB2 phosphorylates Cdc2-Y15 at similar kinetics as it phosphorylates its known substrate Grb2. The ErbB2 kinase domain is sufficient to directly and specifically phosphorylate Cdc2-Y15. Increased Cdc2-Y15-p in ErbB2-overexpressing cells corresponds with delayed M phase entry. Expressing a nonphosphorylatable mutant of Cdc2 renders cells more sensitive to taxol-induced apoptosis. Thus, ErbB2 membrane receptor tyrosine kinase can confer resistance to taxol-induced apoptosis by directly phosphorylating Cdc2, which functions in the nucleus. Increased Cdc2-Tyr15 phosphorylation was also found in ErbB2-overexpressing tumors from breast cancer patients. Thus, increased Cdc2-Tyr15 phosphorylation may be a pertinent cell-cycle checkpoint defect in ErbB2-overexpressing breast cancers that is involved in taxol resistance.

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AKT INHIBITS C-MYC-MEDIATED APOPTOSIS IN MAMMARY EPITHELIAL CELLS: A MECHANISTIC INVESTIGATION

Danica Ramljak, Christine M. Coticchia, Tagvor G. Nishanian, and Robert B. Dickson

Department of Oncology, Lombardi Cancer Center, Georgetown University, Washington, DC 20007

ramljakd@georgetown.edu

This project aims to uncover the mechanism through which Akt, a major survival/proliferation signaling molecule, inhibits c-Myc-mediated apoptosis in cellular models of human breast cancer. Previously we showed that c-Myc-mediated apoptosis is inhibited through survival signaling pathways incident from the epidermal growth factor receptor (EGFR), both in vivo and in vitro. However, it was not clear what survival pathways downstream of EGFR inhibit c-Myc-mediated apoptosis. We and others have established that activation of EGFR promotes survival in association with upregulation of Bcl-xL, through an unknown mechanism. Here we have chosen pro-apoptotic, c-Myc-overexpressing murine mammary epithelial cells (MMECs) derived from MMTV-c-Myc transgenic mouse tumors. Using these cells (Myc83) we show that EGFR activation promotes survival in parallel with activation of Akt and Erk1/2, and upregulation of Bcl-xL. In addition, blockade of EGFR kinase activity and the PI3-K/Akt and MEK/Erk pathways, with pharma-cological inhibitors resulted in a significant induction of cellular apoptosis, paralleled by a down- regulation of both Akt and Erk1/2 proteins. Consistent with a survival-promoting role of Akt, we observed that constitutively activated Akt (Myr-Akt) promoted survival of Myc83 cells, following the inhibition of EGFR tyrosine kinase activity. In addressing possible downstream effectors of Akt-mediated survival, we detected significant upregulation of Bcl-xL protein in Myc83-Myr-Akt cells, suggesting that Bcl-xL may be a novel target of Akt. Presently, the mechanism by which c-Myc-sensitizes mammary cells to apoptosis is unknown. Using both Myc83 and MCF10A-Myc cells (immortalized human mammary epithelial cells overexpressing c-Myc), we have implicated the mitochondrial pathway and cytochrome c. However, it seems that in addition to cytochrome c release, there is a requirement for an additional apoptotic stimulus, currently under investigation. The impact of Akt and Bcl-xL on c-Myc-mediated apoptosis is also being studied. Our studies are the first to examine the c-Akt/c-Myc interaction in models of breast cancer and will provide a rational basis for future potential therapeutic approaches directed toward Akt signaling in breast cancers overexpressing c-Myc.

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DO CALCIUM-ACTIVATED CHLORIDE CHANNELS SUPPRESS TUMORIGENESIS BY PROMOTING APOPTOSIS?

Randolph C. Elble, Janel Beckley, and Bendicht U. Pauli

Department of Molecular Medicine and Cancer Biology
Program, Cornell University, Ithaca, NY 14853

rce3@cornell.edu

The role of plasma membrane ion channels in cell proliferation and arrest, and therefore tumor biology, is only recently coming to light. Electrophysiological and pharmacological evidence from multiple systems suggests that cell cycle progression is promoted by potassium channels but antagonized by chloride channels. We have discovered a new family of calcium-activated chloride channels (CLCA) whose differential regulation in normal, apoptotic, and transformed mouse mammary epithelial cells suggests that channel function is proapoptotic and antineoplastic. In mouse, a pair of these channels is expressed but under opposing conditions. mCLCA1 predominates over mCLCA2 under normal physiological conditions, but mCLCA2 gene expression is strongly induced by apoptotic stress both in involuting mammary gland and in cultured HC11 mammary epithelial cells. Consistent with an apoptosis-promoting role, splicing of mCLCA2 is disrupted in apoptosis-resistant tumor cell lines and in HC11 cells selected for resistance to anoikis. Unexpectedly, mCLCA1 message is also downregulated in these cells, by at least thirtyfold. These results suggest that both genes antagonize survival of mammary tumor cells by sensitizing them to anoikis. When MCF7 or HEK293 tumor cells were transfected with plasmids encoding either mCLCA1 or mCLCA2, colony formation was greatly reduced relative to a vector transfected control, demonstrating that CLCA overexpression is deleterious to tumor cell survival. Furthermore, mammary epithelial cells overexpressing mCLCA2 had twice the rate of apoptosis of normal cells when subjected to serum-starvation and formed multinuclear giants at a high frequency in normal culture, suggesting that mCLCA2 can promote either apoptosis or senescence. Parallel analysis of human tumor samples and cell lines by RT-PCR have shown a nearly universal downregulation of human CLCA genes hCLCA2 and hCLCA4 in breast cancer, while transfection of hCLCA2 into MDA-MB-231 was found to inhibit tumor establishment and growth in mouse. These studies reveal a previously unappreciated mode of tumor suppression and suggest a new avenue of attack on the cancer cell: re-establishment of CLCA gene function.

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THE AUTOCRINE INDUCTION OF TRAIL BY GENOTOXINS

Aaron Christopher Spalding

University of Colorado Health Sciences Center,
Denver, CO 80262

gary.johnson@uchsc.edu

TNF related apoptosis inducing ligand, TRAIL, is a recently cloned cytokine that has been shown to induce apoptosis in a synergistic fashion with chemotherapeutic agents on several cancer cell lines. Xenografts of several carcinoma cell lines demonstrate TRAIL and chemotherapy to cause complete regression of established tumors. Etoposide, a topoisomerase type II inhibitor, induces the upregulation of TRAIL in several breast and lung carcinoma lines. Analysis of human lung tumors demonstrates TRAIL mRNA expression is significantly decreased in tumor relative to autologous non-tumor lung tissue. Genotoxin-induced apoptosis of human cancer cells correlates with TRAIL surface expression. Expression of TRAIL and its death receptor, DR5, is regulated by NFκB. NFκB inhibition results in aggressive growth and chemotherapy resistance of H157 human lung squamous carcinoma. Gene profiling of H157 cells demonstrates that NFκB regulates the expression of both pro- and anti-apoptotic proteins including inhibitors of apoptosis (IAPs) in addition to DR5 and TRAIL. It is the predominance of the opposing NFκB-dependent signals that dictates the cell's decision to survive or die. Loss of TRAIL expression in human lung cancer provides a means by which tumors can avert programmed cell death. DcR1, a GPI-linked TRAIL receptor, has been proposed to confer TRAIL resistance by serving as a membrane bound TRAIL trap. DcR1 expression inhibits caspase 8 and BID cleavage as well as JNK phosphorylation induced by etoposide and TRAIL. DcR1, however, also induces potent survival signals. Restoration of DcR1 in breast carcinoma cells to physiologic levels induces Akt activation in a src family kinase dependent manner. DcR1 expression confers resistance to mitochondria insult and apoptosis induced by currently utilized chemotherapeutic agents as well as to Antimycin A, a BH3 domain homologue that targets mitochondria directly. These data indicate that in addition to inhibiting DR4 and DR5 signaling by binding TRAIL, DcR1 also influences cell survival by preserving mitochondrial integrity. DcR1 is a regulator of the apoptosis module whose expression enhances cell survival and confers resistance to chemotherapy. This work demonstrates the importance of TRAIL and its receptors in neoplasia response to DNA damaging agents.

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THE ROLE OF THE PROHIBITIN GENE IN APOPTOSIS OF BREAST CANCER CELLS

Fusaro G.,[1] Wang S., and Chellappan S.[2]

[1]Columbia University,
[2]H. Lee Moffitt Comprehensive Cancer Center

fusarog@moffitt.usf.edu

Prohibitin, a potential tumor suppressor protein, was originally identified by its ability to induce G1/S arrest in human diploid fibroblasts. The prohibitin gene was subsequently shown to be mutated in several sporadic breast tumors. Studies from our lab have since shown that prohibitin binds Rb and inhibits the activity of the E2F family of transcription factors. Our studies show that prohibitin protects breast cancer cells from apoptosis induced by the chemotherapeutic drug camptothecin and may affect p53 function.

Initial experiments in B cells suggest that prohibitin may be protective against cytotoxic activity induced by particular drugs. A human B cell line (Ramos) stably over-expressing prohibitin was treated with camptothecin, Taxol, 5-fluorouracil, or tamoxifen, and the amount of apoptosis was determined. Surprisingly, cells overexpressing prohibitin exhibit about 50% less death upon treatment with camptothecin, a topoisomerase I inhibitor, compared to the parental cell line. Furthermore, studies using breast cancer cell lines also indicate a protective role of prohibitin during camptothecin treatment. BT 549 cells, which express high levels of endogenous prohibitin, exhibit 50% less death from camptothecin than ZR 751 cells, which have low levels. In addition, prohibitin protein levels increased after camptothecin treatment, while Rb was completely degraded. Correspondingly, the amount of E2F in complex with Rb as well as p107 and p130 decreased. Cyclin D levels remain constant, whereas cyclin E protein and message levels increase. Concomitant with dissociation with its repressors, E2F transcriptional activity increases in response to this drug, but this increase is attenuated in cells overexpressing prohibitin. Plus, we find that prohibitin may aid in activating p53 transcription in breast cancer cells. Likewise, we also find that prohibitin may cooperate with p53 to repress E2F activity in breast cancer cells. It appears that prohibitin may intersect both the Rb/E2F pathway and the p53 pathway, providing a link between proliferation and growth control. Our studies are thus elucidating the mechanisms whereby prohibitin affects the chemotherapeutic response and may help in directing therapeutic strategies for patients with breast cancer.

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BCL-G, A NOVEL PRO-APOPTOTIC MEMBER OF THE BCL-2 FAMILY

Bin Guo, Adam Godzik, and John C. Reed

The Burnham Institute

bguo@burnham.org

Resistance to chemotherapy remains the major problem in the treatment of breast cancer. Resistance to drug-induced apoptosis has been implicated in poor clinical responses to chemotherapy. The objective of this project is to study the molecular mechanisms of novel Bcl-2 family proteins in the regulation of apoptosis.

FISH analysis and loss of heterozygosity (LOH) studies have previously delineated a 600 kb region on chromosome 12p12.3 (between ETV6/TEL gene and D12S358), which is subjected to frequent LOH among childhood acute lymphoblastic leukemia, prostate cancer, and ovarian cancers. A novel pro-apoptotic member of the Bcl-2 family, Bcl-G, was identified and mapped within this region adjacent to LRP6 gene. Bcl-G gene encodes two proteins through alternative mRNA splicing: Bcl-GL (long) and Bcl-GS (short) consisting of 327 and 252 (length) amino-acids, respectively. Bcl-GL and Bcl-GS are identical in their first 226 amino-acids but diverge thereafter. Among the Bcl-2 Homology (BH) domains previously recognized in Bcl-2 family proteins, the BH3 domain is found in both Bcl-GL and Bcl-GS, but only the longer Bcl-GL protein possesses a BH2 domain. Bcl-GL mRNA is expressed widely in normal human tissues, whereas Bcl-GS mRNA was found only in testis. Over-expression of Bcl-GL or Bcl-GS in cells induced apoptosis, but Bcl-GS was far more potent than Bcl-GL. Apoptosis induction by Bcl-GS depended on the BH3 domain, and was suppressed by co-expression of anti-apoptotic Bcl-XL protein. Bcl-XL also co-immunoprecipitated with Bcl-GS but not with mutants of Bcl-GS in which the BH3 domain was deleted or mutated and not with Bcl-GL. Bcl-GS was predominantly localized to cytosolic organelles whereas Bcl-GL was diffusely distributed throughout the cytosol. A mutant of Bcl-GL in which the BH2 domain was deleted displayed increased apoptotic activity and co-immunoprecipitated with Bcl-XL, suggesting that the BH2 domain auto-represses Bcl-GL.

Future studies will address the mechanisms of Bcl-G activation in breast cancer cells during responses to chemotherapeutic drugs. These studies will provide insights into the mechanisms of Bcl-G action in regulating apoptosis and improve understanding of mechanisms of drug resistance in breast cancer.

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A RADIATION-INDUCIBLE HUMAN APOPTOTIC REGULATOR WITH HOMOLOGY TO *DROSOPHILA* REAPER

Christopher Holley and Sally Kornbluth

Duke University, Department of Pharmacology
and Cancer Biology, Durham, NC 27710

cholley@duke.edu

Currently, one of the most important options for treating breast cancer is therapeutic radiation. In principle, radiation preferentially kills rapidly dividing cells by inducing the cell death process known as apoptosis. Although much is known about how human cells detect damage due to radiation (especially via p53), very little is known about precisely how that signal results in apoptosis. Use of model organisms such as the fruit fly Drosophila has led to insights into the induction of apoptosis following ionizing radiation. In the fly (as in humans), damage due to radiation is sensed by p53. Furthermore, genetic studies in the fly have shown that the reaper gene is an important downstream effector for inducing apoptosis in response to ionizing radiation. We therefore have sought a human homolog to this gene in order to better understand the induction of apoptosis following ionizing radiation.

The approach we took was to search publicly available databases for human reaper homologs and to synthesize a cDNA library from irradiated human cells. Candidate genes would be cloned from the custom library which contains radiation-induced transcripts. Corresponding protein products would be tested for its ability to induce apoptosis. The candidate proteins would also be tested for its ability to bind to Scythe and a family of proteins known as inhibitors of apoptosis, both of which are known Reaper-interactors.

The results to date are that a human Reaper homolog has been identified, and it has some but not all of the properties of fly Reaper. The human Reaper homolog was induced by ionizing radiation and bound to Scythe, but it did not interact with the inhibitors of apoptosis. On its own, the human Reaper homolog is a weak inducer of apoptosis, but it appears to be complemented by other factors such as Smac/Diablo to fully recapitulate the degree of apoptosis induced by Reaper.

We conclude that the human Reaper homolog is a relevant component of the signaling pathway leading to apoptosis in response to ionizing radiation. Further investigation of the human Reaper pathway will hopefully lead to an even better understanding of how radiation therapy leads to apoptosis.

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RECIPROCAL REGULATION OF APOPTOSIS AND SENESCENCE IN BREAST CELLS TREATED WITH ADRIAMYCIN

Shawn E. Holt, Catharine I. Dumur, Andrea Ferreira-Gonzalez, David A. Gewirtz, and Lynne W. Elmore

Department of Pathology, Department of Pharmacology and Toxicology, Department of Human Genetics, and the Massey Cancer Center, Medical College of Virginia at Virginia Commonwealth University, Richmond, VA

seholt@hsc.vcu.edu

It is unclear why chemotherapeutic regimens that are highly effective at inducing apoptosis in many types of tumor cells, often prove ineffective for breast cancer cells. Since MCF-7 human breast cancer cells undergo a senescence-like response following acute adriamycin exposure, we and others have proposed that senescence and apoptosis may be two mutually exclusive phenomena. To test this hypothesis, we have compared the gene expression profiles of untreated vs. adriamycin treated MCF-7 using Affymetrix oligonucleotide arrays (chip set HG_U95Av2), as well as with normal mammary epithelial cells. Consistent with a growth arrested state, treated cells expressed nearly undetectable levels of cyclins A, B, E2, F as well as low levels of PCNA and cdc 2. In agreement with Western blotting data, p21waf-1 remained elevated (11.9-fold induction) four days after treatment, while p53 expression was back to levels observed in the untreated parental cells. Despite only rare TUNEL positive cells following acute adriamycin exposure, a number of apoptotic modulators were induced by adriamycin, with the number of pro-apoptotic mediators (e.g., FAS/APO-1, bax, TRAIL receptor 2, BID, c-jun, MEK kinase, TNFR-related DR6, andJAK-1) outnumbering the anti-apoptotic regulators (e.g., bcl-2, galectin-3; and IGFBP-5). Collectively, these data indicate that senescence and apoptosis are not mutually exclusive pathways, and that MCF-7 cells are unable to successfully execute apoptotic cell death following adriamycin exposure.

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PHOSPHORYLATION OF CEACAM1-4S INDUCES LUMEN FORMATION BY APOPTOSIS IN TRANSFECTED MCF-7 MAMMARY CARCINOMA CELLS

Julia Kirshner,[1] Charng-Jui Chen,[2] Ping-Fang Liu,[1] Jie Huang,[3] and John E. Shively[2]

[1]Graduate School of the City of Hope and Beckman Research Institute, Duarte, CA 91010; [2]Division of Immunology, Beckman Research Institute of the City of Hope, Duarte, CA 91010; [3]School of Medicine, University of California San Diego, La Jolla, CA 92093

jkirshner@coh.org

The cell-cell adhesion molecule carcinoembryonic antigen cell adhesion molecule 1 (CEACAM1) is a cell surface glycoprotein and a member of the CEA gene family. Normal mammary epithelial cells express CEACAM1 in a polarized, lumenal orientation and CEACAM1 has been shown to play an essential role in lumen formation in a mammary morphogenesis model system using MCF10F cells. Here we show that MCF7 mammary carcinoma cells, which do not express CEACAM1 or make lumens when grown in Matrigel, are restored to a normal morphogenic program when transfected with CEACAM1-4S, the short cytoplasmic isoform of CEACAM1. Specific staining with anti-cytokeratin 18 antibody or MitoSensor dye demonstrated that the lumens are formed by apoptosis of the central cells within the alveolar structures. Moreover, apoptotic cells within the lumen were the same cells that expressed CEACAM1-4S, as demonstrated by visualization of CEACAM1-4S-exoGFP fusion protein expressed in MCF7 cells together with MitoSensor staining. Furthermore, alterations in phosphorylation patterns of CEACAM1-1 lead to diminished lumen formation or the death of the entire acinus. Since Thr(423) and Ser(425) are putative protein kinase C phosphorylation sites, we mutated these residues to Asp or Ala conferring pseudophosphorylation and null mutations respectively. All of the Ala null mutants did not form lumens in Matrigel. In contrast, the T423D mutation lead to colony death for cells grown in Matrigel but not on plastic. Strikingly, the T423D,S425D double mutation was lethal for cells grown on plastic, with cell death occurring as early as 24 hours post transfection. Transfection of MCF7 cells with CEACAM1-4L, the long cytoplasmic isoform of CEACAM1 resulted in massive death of these cells when grown in Matrigel but not on plastic. These data suggest that both isoforms are capable of delivering a death signal, but CEACAM1-4S, the predominant isoform in normal mammary epithelial cells, is probably the physiologically relevant isoform for these cells. Taken together these data demonstrate that CEACAM1-4S can revert tumor cells to a normal morphogenic program by promoting apoptosis of the cells in the center of the acini that lack contact with the extracellular matrix.

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TIMP-1 REGULATION OF APOPTOSIS IN HUMAN BREAST EPITHELIAL CELLS

Xu-Wen Liu, Rafael Fridman, and Hyeong-Reh C. Kim

Department of Pathology, Wayne State University
School of Medicine, Detroit, MI 48201

hrckim@med.wayne.edu

The importance of apoptosis in normal development and pathogenesis has been well recognized, and explosive progress towards dissecting its commitment step has been made during the past decade. Mitochondria, Apaf-1, caspase, and bcl-2 family members play central roles in the commitment step. However, it is still unclear how upstream cell survival pathways regulate apoptosis. It is also unknown whether the bcl-2 family members have any effect on the upstream survival pathways. Our studies demonstrate that the anti-apoptotic gene product bcl-2 greatly induces expression of the tissue inhibitor of metalloproteinase-1 (TIMP-1) in human breast epithelial cells. Surprisingly, we found that TIMP-1, like bcl-2, is a potent inhibitor of apoptosis induced by a variety of stimuli. Functional studies indicate that TIMP-1 inhibits a classical apoptotic pathway mediated by caspases, and that focal adhesion kinase (FAK)/PI 3-kinase and mitogen activated protein kinase (MAPK) are critical for TIMP-1-mediated cell survival. We show specific association of TIMP-1 with the cell surface. Consistently, a 150-kDa surface protein was identified in MCF10A cells that specifically binds TIMP-1. Taken together, we hypothesize that TIMP-1 binding on the cell surface induces a cell survival pathway that regulates the common apoptosis commitment step. Our studies address a new paradigm in the regulation of apoptosis by an extracellular molecule TIMP-1, and also greatly enhance our understanding of TIMP-1's pleiotropic activity in many physiological and pathological processes. This information may also be useful in designing more rational therapeutic interventions aimed at modulating the anti-apoptotic activity of TIMP-1.

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GALECTIN-3 TRANSLOCATES TO THE PERINUCLEAR MEMBRANES AND INHIBITS CYTOCHROME C RELEASE FROM THE MITOCHONDRIA: A ROLE FOR SYNEXIN IN GALECTIN-3 TRANSLOCATION

Fei Yu,[1] Russell L. Finley, Jr.,[2] Avraham Raz,[1] and Hyeong-Reh Choi Kim[1]

[1]Department of Pathology, and
[2]Center for Molecular Medicine and Genetics,
Wayne State University School of Medicine, and
Karmanos Cancer Institute, Detroit, MI 48201

hrckim@med.wayne.edu

Galectin-3 is a multifunctional oncogenic protein found in the nucleus, cytoplasm and also extracellular milieu. Although we have demonstrated an anti-apoptotic activity of galectin-3 in response to a variety of apoptotic stimuli (Cancer Research 59:4148, 1999; Carcinogenesis 21:1941, 2000; Am. J. Pathology 159:1055, 2001), neither the functional site nor the mechanism of how galectin-3 regulates apoptosis is known. In the present study, we examined the subcellular localization of galectin-3 during apoptosis and investigated its anti-apoptotic actions. We report that galectin-3 translocates into the perinuclear membrane following a variety of apoptotic stimuli. Confocal microscopy and biochemical analysis revealed that galectin-3 is enriched in the mitochondria and prevents mitochondrial damage and cytochrome c release. Using a yeast two-hybrid system we screened for galectin-3 interacting proteins that regulate galectin-3 localization and anti-apoptotic activity. Synexin, a Ca2+- and phospholipid-binding protein, was one of the proteins identified. We confirmed direct interaction between galectin-3 and synexin by GST-pulldown assay in vitro. We showed that galectin-3 fails to translocate to the perinuclear membranes when the expression of synexin is downregulated using an oligodeoxyribonucleotide complementary to the synexin mRNA, suggesting a role for synexin in galectin-3 trafficking. Furthermore, synexin downregulation abolished anti-apoptotic activity of galectin-3. Taken together, the present study suggests that synexin mediates galectin-3 translocation to the perinuclear mitochondrial membranes, where it regulates mitochondrial integrity critical for apoptosis regulation (J. Biol. Chem. In press M200154200, 2002).

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SYNTHESIS OF PRO-APOPTOTIC FORM OF CLUSTERIN IN BREAST CANCER CELLS

Konstantin S. Leskov, Jing Li, Dmitri Klokov, and David A. Boothman

Case Western Reserve University

ksl9@po.cwru.edu

Clusterin (CLU) is an ionizing radiation (IR)-induced protein that is reported to have both pro- and anti-apoptotic functions. The major form of the CLU protein is secreted (sCLU), and is induced by low doses of IR (>2 cGy). Several groups have shown that sCLU acts as a protector against apoptosis.

However, the existence of another, nuclear form of CLU (nCLU) was proposed and reported by us (Yang et. al., PNAS, 2000). We demonstrated that nCLU induced caspase-3-independent cell death upon forced over-expression in MCF7:WS8 breast cancer cells. We also demonstrated that the C-terminal coiled-coil domain of nCLU was responsible for cell death. Our recent data indicate that the N-terminal coiled-coil domain of nCLU bound to the C-terminal coiled-coil domain, promoting either intra-molecular folding or oligomerization. We showed that endogenous nCLU protein was induced in the nuclei of IR-treated cells by higher doses of IR (1 Gy), and we hypothesized that nCLU protein induction may be necessary for the elimination of severely damaged cells. We recently determined the mechanisms for nCLU synthesis. We isolated a cDNA for nCLU from IR-treated MCF7:WS8 breast cancer cells. This nCLU mRNA was produced by splicing together exons I and III, and eliminating exon II, which contained the first AUG codon and the endoplasmic reticulum (ER)-targeting signal of sCLU. In the result of this alternative splicing, translation started from the second in-frame AUG codon positioned in exon III leading to the production of the nCLU protein. We are currently investigating the expression of nCLU in mammalian cells, its functions regarding apoptosis, and its responses to IR, including regulation of splicing and translation initiation. We propose that CLU RNA message undergoes alternative splicing resulting in pro- and anti-apoptotic form of the protein.

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THE ROLE OF APOPTOSIS IN METASTATIC INEFFICIENCY OF BREAST CANCER

G. N. Naumov, P. M. Weinmeister, I. C. MacDonald, S. M. Wilson, A. C. Groom, and A. F. Chambers

University of Western Ontario and London Regional Cancer Centre, London, Ontario, Canada

gnaumov@hotmail.com

Metastasis is a major contributor to death from breast cancer. However, it is an inefficient process where only few metastatic tumors result from many cells shed by the primary tumor. It is uncertain at which step(s) of the process the tumor cells are lost and what factors contribute to this loss. The overall goal of this project is to investigate the role of apoptosis in metastatic inefficiency of breast cancer cells. As a model for studying mechanisms of metastasis we compared two murine mammary carcinoma cell lines: D2A1 cells (highly metastatic) and D2.0R cells (poorly metastatic). Our specific goals were to learn how the cell lines differ in survival kinetics in a secondary site, and to determine the role played by apoptosis in the metastatic vs. poorly metastatic cell line. We used a combination of in vivo videomicroscopy and detailed kinetic analyses to quantify the survival and growth of these cells at various times after intraportal injection to target them to mouse liver. Using a cell accounting assay and 50 mm thick formalin fixed tissue sections we quantified survival of single cells and metastases immediately after injection or 3, 10, 14, 18, or 21 days later. We found that: Initially 85% of injected cells (both cell types) survived in the liver microcirculation. By day 10, 65% of injected D2A1 cells remained as solitary undivided cells. However, a small sub-population of the injected cells (0.623%) had started proliferating. Not all of these early metastases continued to grow, with the number of early metastases decreasing by a factor of 100 between days 10 and 14, although total metastatic burden increased dramatically over the same period. To examine possible mechanisms for the loss of D2A1 solitary cells and early metastases between 10 and 14 days we quantified apoptosis and proliferation, by TUNEL and Ki67 staining, both in solitary cells and in the metastases. At most time points apoptosis levels in solitary cells were low (<1%). However, a 4-fold increase in apoptotic solitary cells was found at day 14, and this peak of apoptosis occurred at the time of greatest single cell loss. A similar peak of apoptosis was detected within the early metastases at day 14, at the time of greatest loss of metastases. In contrast, a surprisingly high proportion of the injected D2.0R cells remained as solitary undivided cells for up to 11 weeks after injection. These cells were judged to be dormant by their consistently low levels of both apoptosis and proliferation, as assessed in histological sections by TUNEL and Ki67. However, when recovered from the tissue, at day 77, such cells were able to grow in vitro and to form tumors when re-injected in the mammary fat pad, confirming the persistence of viable cells. This study thus identified specific steps of metastasis in which apoptosis can play a role, in particular: at initiation of growth and maintenance of early metastasis growth. Moreover, we found that poorly metastatic cells can persist in large numbers over long periods of time without any significant indication of growth or death. This study thus suggests that highly vs. poorly metastatic cells may differ in their susceptibility to in vivo apoptotic destruction. It also raises the possibility that solitary dormant cells can persist for long periods of time in secondary sites, and may contribute to tumor recurrence after a period of clinical dormancy.

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MECHANISMS OF REAPER-INDUCED APOPTOSIS

Michael R. Olson and Sally Kornbluth

Duke University Medical Center

mro@duke.edu

Apoptosis, or programmed cell death, is an energy-dependent process of cell suicide shown to be critical in many physiological venues including development, immune development, immune response, and tissue homeostasis. In addition, the development of tumors (especially soft tumors as in the breast) depends on the cell's ability to escape the apoptotic response normally triggered in response to cell damage. Although the physical characteristics of apoptosis have been well characterized (membrane blebbing, nuclear condensation, DNA degradation), characterization of the exact mechanism is still ongoing. Perhaps the most interesting players in the pathway are its regulators—both the inducers of apoptosis and their corresponding inhibitors. Many of each class have been identified. The gene reaper from Drosophila melanogaster, is one of three genes at a genetic locus shown to be essential for developmental and radiation-induced apoptosis in the fruit fly. More intriguing is the observation that reaper is induced by gamma irradiation in a p53-dependent manner. Moreover, recent studies in cell culture demonstrate that a cell line derived from breast carcinoma (MCF-7) is sensitive to reaper-induced apoptosis.

Using a cell-free extract system, our lab has shown that Reaper can induce the release of the proapoptotic molecule, cytochrome c, from the intermembrane space of mitochondria. This is an activity normally associated with the Bcl-2 family of molecules, a class of proteins with well-established importance to breast and other cancers. We have recently demonstrated that Reaper can interact directly with mitochondria, and that this interaction appears essential for Reaper function. Mutants of Reaper which fail to localize to mitochondria fail to induce apoptosis in cultured cells. Yet these Reaper mutants still retain many of the biochemical functions of wild-type Reaper, including the ability to bind Inhibitor of Apoptosis (IAP) proteins. Therefore, mitochondrial localization of Reaper to mitochondria is necessary for its function, underscoring yet again the importance of mitochondria in the regulation of apoptosis and reinforcing the notion that inducing cell death may be best effected by manipulation of signaling events at the mitochnondrial surface.

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NOVEL ROLE FOR HUMAN RAD21 COHESIN IN APOPTOSIS

Debananda Pati and Sharon E. Plon

Department of Pediatrics,
Baylor College of Medicine, Houston, TX

pati@bcm.tmc.edu

Sister chromatid cohesion during DNA replication plays a pivotal role in accurate chromosome segregation in the eukaryotic cell cycle. Rad21 is one of the major cohesion subunits keeping sister chromatids together until anaphase, when proteolytic cleavage by separase allows the chromosomes to separate. Mitotic cleavage sites in Rad21 in yeast and humans have been mapped. Here we show that cleavage of human Rad21 (hRad21) also occurs during apoptosis and is induced by various agents including DNA damaging (topoisomerase inhibitors) as well as non-DNA damaging agents (cycloheximide). Recognition by affinity-purified polyclonal and monoclonal antibodies to hRad21 confirms the identities of the cleavage products. We have biochemically mapped the apoptotic cleavage site in hRad21, which is distinct from mitotic cleavage sites previously described. The apoptotic cleavage site is conserved among vertebrates, and cleavage is likely to be mediated by a novel caspase. Cleavage of hRad21 appears to be an early event in the apoptotic pathway. Induction of apoptosis in multiple human cell lines results in the early (4 hours post insult) generation of 64 kDa and 60 kDa hRad21 cleavage products. Although hRad21 is a nuclear protein, the cleaved 64 kDa carboxy-terminal product is found predominantly in the cytoplasm, and overexpression of the cleavage product results in apoptosis. While hRad21 is cleaved by various apoptotic agents, equivalent doses of ionizing radiation in cells resistant to apoptosis do not generate cleavage bands; thus, hRad21 cleavage is not a simple byproduct of DNA damage. Given the role of hRad21 in chromosome cohesion, the cleaved C-terminal product and its translocation to the cytoplasm may act as a nuclear signal for apoptosis. A role for hRad21 in apoptosis is further strengthened by identification of a number of genes involved in apoptosis as interactors of hRad21 in a two-hybrid assay. In summary, in addition to previously described functions of hRad21 in chromosome segregation and DNA repair, cleavage of the protein and its traslocation to the cytoplasm is an early event in the apoptotic pathway. These results provide the framework to identify the physiologic role of hRad21 in the apoptotic response of normal and malignant cells.

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APOPTOTIC REGULATION THROUGH THE ADAPTER PROTEIN CRK

D. Ashley Richardson, Jesse J. Smith, and Sally Kornbluth

Duke University Medical Center, Durham, NC 27710

dar11@duke.edu

Apoptosis is a cellular suicide program whereby individual cells are destroyed while maintaining the integrity of surrounding tissue. Such targeted cell death is essential for embryogenesis and tissue homeostasis. However, apoptotic evasion enables tumorigenesis by promoting the survival of hyperproliferative cells. A more detailed characterization of apoptotic mechanisms and their deregulation during carcinogenesis is essential for the development of more specific and effective cancer therapies. All apoptotic signals converge on a conserved series of biochemical events that produce the apoptotic phenotype. These events include release of mitochondrial cytochrome c, caspase activation, and activation of apoptotic nucleases. Although the mechanism of certain apoptotic pathways, such as the Fas/TNFα death receptor pathway, are well characterized, the specific signaling route from initiation to death remains poorly understood for numerous apoptotic pathways.

We are currently focused on the role of the adapter protein Crk in apoptotic signaling. Previous research conducted in our laboratory demonstrated that Crk is required for apoptosis in Xenopus egg extracts. Immunodepletion of endogenous Crk or depletion on resins made from Crk SH2 or amino terminal SH3 domains causes egg extracts to lose apoptotic activity. The novel observation of an adapter protein's involvement in apoptosis prompted us to elucidate the mechanism of Crk coupling to death pathways.

Recently, we have determined that Crk's subcellular localization and association with Wee1 is necessary for its role in apoptotic signaling. We have observed by yeast two-hybrid and immunoblot analysis that Crk interacts with the nuclear export factor Crm1. We have identified a putative nuclear export sequence (NES) within the carboxyl terminal SH3 domain of Crk. Consistent with the hypothesis that Crk shuttles between the nucleus and the cytoplasm, a significant portion of endogenous Crk resides in the nucleus of U2OS cells. A mutant Crk unable to bind Crm1, Crk NES-, displays enhanced Wee1 binding and accelerates apoptosis relative to wild type Crk. Based on these observations, we hypothesize that Crk engages a nuclear proapoptotic pathway regulated by Wee1 association.

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CHROMOSOME 17 (p13.2) CONTAINS GENE/S CONTROLLING FAS-MEDIATED APOPTOSIS IN TRANSFORMED HUMAN BREAST EPITHELIAL CELLS

M. H. Lareef, Q. Tahin, I. H. Russo, G. Mor, J. Song, D. Mihaila, C. M. Slater, and J. Russo

Breast Cancer Research Laboratory, Fox Chase Cancer Center, Philadelphia, PA; and Department of Reproductive Immunology, Yale University, New Haven, CT

mh_lareef@fccc.edu

The human breast epithelial cells (HBEC) BP1E, which have been transformed with the chemical carcinogen benz(a)pyrene (BP), express *in vitro* phenotypes indicative of neoplastic transformation and microsatellite instability at 17p13.2. Transfection of chromosome (chr) 17 to BP1E cells inhibits the transformation phenotypes, an indication that the transfected 17p13.2 region reverts the neoplastic process. This work was designed for determining whether the reversion was mediated by activation of Fas receptors, which are known to induce apoptosis. We used MCF-10F, BP1E, BP1E-11neo, BP1E-17neo, and BP1E-14hygro cell lines for testing their sensitivity to Fas-induced apoptosis. The cells were incubated in triplicate with anti-human Fas monoclonal antibody at the concentrations of 50, 100, 150, and 200ng/ml for 24 hr, and then with MTT; absorbency was measured at 540nm for determination of the percentage of absorbency relative to the control normal mouse IgG antibody. MCF-10F cells and 7 out of 10 BP1E-17neo clones exhibited a dose dependent maximal sensitivity. BP1E cells, BP1E-11neo, BP1E-14hygro, and 3 BP1E-17neo clones were resistant at all doses tested. PCR analysis revealed that the 7 sensitive clones contained the 17p13.1-13.2 regions (markers D17S1852, D17S796, D17S13 and TP53), which was absent in 3 resistant clones. Our data indicate that the Chromosome 17p13.2(D17S796) contain gene or genes that regulates Fas receptor activation. Cloning of this genes/genes could provide tools for gene therapy.

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INTRACELLULAR MASPIN SENSITIZES BREAST CARCINOMA CELLS TO INDUCED APOPTOSIS

Shijie Sheng, Ning Jiang, and Yonghong Meng

Wayne State University School of Medicine

sheng@med.wayne.edu

Maspin, a novel serine protease inhibitor (serpin), suppresses the growth and metastasis of breast tumor in vivo. However, the underlying molecular mechanism is unclear. In the current study, we report the first evidence that endogenous maspin expression in mammary carcinoma cells MDA-MB-435 enhanced staurosporine (STS)-induced apoptosis as judged by the increased fragmentation of DNA, increased proteolytic inactivation of poly-[ADP-ribose]-polymerase (PARP), as well as the increased activation of caspase-8 and caspase-3. In parallel, recombinant maspin did not directly regulate the proteolytic activities of either caspase-3 or caspase-8 in vitro. Consistent with this result, maspin expressing normal mammary epithelial cells underwent more rapid STS-induced apoptosis as compared to breast carcinoma cells. Interestingly, maspin transfectant cells did not undergo spontaneous apoptosis in the absence of STS. Moreover, neither purified maspin protein added from outside nor endogenous maspin secreted to the cell culture media sensitized cells to STS-induced apoptosis. To investigate the structural determinants of maspin in its apoptosis-sensitizing effect, MDA-MB-435 cells were also transfected with maspin/PAI-1 and PAI-1/maspin chimeric constructs resulting from swapping the N-terminal and the C-terminal domains between maspin and PAI-1 (plasminogen activator inhibitor type 1). The resulting stable transfectant clones expressing maspin/PAI-1 and PAI-1/maspin, respectively, did not undergo spontaneous apoptosis, and were similarly inhibited as maspin transfectant cells in motility assay. Interestingly, however, expression of both maspin/PAI-1 and PAI-1/maspin in MDA-MB-435 cells failed to sensitize these cells to STS-induced apoptosis. Taken together, our evidence provides new insights into the complex molecular mechanisms of maspin that may suppress breast tumor progression not only at the step of invasion and motility, but also by regulating tumor cell apoptosis. The sensitizing effect of maspin on apoptosis is to be contrasted by the pro-survival effect of several other serpins.

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ANALYSIS OF PRO-APOPTOTIC AND ANTIANGIOGENIC ACTIVITY OF CC3 IN BREAST CANCER CELLS

Frank King and Emma Shtivelman

Cancer Research Institute,
University of California, San Francisco

shtivelman@cc.ucsf.edu

Development of aggressive and metastatic phenotypes in tumors largely depends on acquisition by tumor cells of ability to support angiogenesis and resist apoptosis. Human gene CC3 is able to negatively affect both of these features of metastatic cells. CC3 is a metastasis suppressor of variant small cell lung carcinoma (vSCLC) and a mouse melanoma in vivo. We have identified two likely mechanisms whereby CC3 acts as a metastasis suppressor. First, forced expression of CC3 restores apoptotic responses of tumor cells to a wide variety of signals. Since the ability to resist apoptosis is an essential part of metastatic phenotype, the pro-apoptotic function of CC3 is likely to contribute to suppression of metastasis. Second, we have discovered that expression of CC3 in tumor cells results in suppression of their angiogenic activity in vitro. Enforced expression of CC3 in tumor cells leads to reduced expression of angiogenic stimulatory factors and increase in antiangiogenic factor production. Since angiogenesis is necessary for the development of metastasis, ability of CC3 to suppress angiogenesis is crucial in its metastasis suppressing ability.

We have introduced expression of CC3 into two breast carcinoma cell lines that were derived from metastatic tumors. By using assays of in vitro angiogenesis we have found that these cell lines do not produce high levels of angigenic factors and are poor stimulators of angiogenesis in vitro. Therefore it is not possible to discern any effect of enforced CC3 expression in terms of suppression of angiogenesis. However, introduction of CC3 resulted in suppression of metastasis in vivo and predisposition to apoptosis.

To understand the mechanism of induction of apoptotic sensitivity by CC3 we have employed biochemical analysis of this protein in vivo. We will present results showing that CC3 exists in cells as a part of a protein complex and describe the other components of this complex identified by mass-spectometry analysis. The analysis of CC3 complexes will provide clues to the mechanisms underlying its metastasis-suppressing function in breast cancer.

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IDENTIFICATION OF GENES THAT INHIBIT TRAIL-INDUCED APOPTOSIS

**Lianghua Bin, Xiaoyan Li,
Liang-Guo Xu, and Hong-Bing Shu**

National Jewish Medical and Research Center

shuh@njc.org

TRAIL is a tumor necrosis factor family member that can specifically induce apoptosis of cancer cells but not of normal cells. However, some cancer cells are resistant to TRAIL-induced apoptosis. Previous studies indicated that the protein synthesis inhibitor cycloheximide could convert TRAIL resistant cells to sensitive cells, suggesting that TRAIL resistant cells but not sensitive cells express one or more short lived proteins that can inhibit TRAIL-induced apoptosis. We have developed an expression cloning approach to screen for such TRAIL inhibitory genes. This approach has identified ~50 candidate clones. Among them, 17 encode for the short splice form of Casper/c-FLIP (Casper-S). Overexpression of Casper-S conferred resistance to TRAIL sensitive cells. Furthermore, Casper knock-out embryonic fibroblasts were highly sensitive to TRAIL, while re-introduction of Casper-S restored resistant to TRAIL-induced apoptosis. These results suggest that Casper-S is a major cellular inhibitor of TRAIL-induced apoptosis.

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PROTEASOME BLOCKADE INDUCES APOPTOSIS BY PREVENTING THE DEGRADATION OF ACTIVE CASPASE-3 SUBUNITS

Jeffrey B. Smith, Lei Chen, Zhi Wang, and Lucinda Smith

Department of Pharmacology and Toxicology, University of Alabama at Birmingham, Birmingham, AL 35294-0019

jeff.smith@ccc.uab.edu

Inhibitors of the ubiquitin (Ub)-proteasome system are potential new anticancer agents. The goal of this study was to test the idea that proteasome blockade induces apoptosis by stabilizing active caspase-3 subunits. Caspase-3 is a pivotal executioner protease in apoptosis. Procaspase-3 (p32) is processed by upstream caspases to p12 and p20 subunits which heterodimerize. Concomitant with the formation of the active heterotetramer, p20 is autoprocessed to p17. Treatment of HL-60 cells with lactacystin (Lacta), a selective proteasome inhibitor, exponentially increased caspase-3 hydrolytic activity and induced apoptosis. Immunoblot analysis showed that the Lacta treatment decreased the p32 zymogen and concomitantly evoked the accumulation of the active p17 and p12 subunits. In contrast to caspase-3, Lacta treatment had little or no effect on caspase-8, or -9, or granzyme B activities. To determine whether caspase-3 subunits were degraded by the Ub-proteasome system, human retinoblast 911 cells were transfected with epitope-tagged caspase-3 subunits. Treatment with a proteasome inhibitor for 4-6 h evoked the accumulation of p12, p17, and p20, but had no effect on p32 zymogen. These data suggest that caspase-3 subunits, in contrast to procaspase-3, are unstable due to degradation by the Ub-proteasome system. Mono- to multi-Ub conjugates of p12 and p17 accumulated following proteasome blockade. Mutation of all eight lysines of p12 to arginine stabilized p12 and abolished p12 ubiquitination, as expected for degradation by the Ub-proteasome pathway. Additional mutation analyses indicated that any single lysine or lysine pair was sufficient for ubiquitination of p12. Mono- to multi-Ub conjugates of p12 accumulated in cells that were cotransfected with a caspase inactive (C163A) mutant of p17. The accumulation of p12-Ub conjugates in cells that expressed an excess of the p17 mutant suggests that the Ub-proteasome system can regulate the accumulation of active caspase-3. These findings indicate that caspase-3 subunits can be degraded by the Ub-protesome system in mammalian cells and suggest that Lacta induces apoptosis by stabilizing active caspase-3 subunits.

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NICOTINE INHIBITS DOXORUBICIN-INDUCED APOPTOSIS OF HUMAN BREAST CANCER CELLS IN CULTURE

Yanfei Zhou,[1] Xinbin Gu,[2] Sunny Dhingra,[2] and Rajagopalan Sridhar[1]

[1]Cancer Center and Department of Radiation Oncology, and
[2]College of Dentistry, Howard University,
Washington, DC 20060

sridhar_howard@hotmail.com

In the United States approximately 7.5 % of cancer related deaths can be attributed to smoking[1]. Use of tobacco after a cancer diagnosis decreases survival, promotes recurrence, field cancerization and decreases the efficacy of cancer therapy[2]. The chemotherapeutic drug doxorubicin induces apoptosis in cancer cells. The effect of nicotine on doxorubicin-induced apoptosis was evaluated *in vitro* using MCF-7 human breast cancer cell cultures. Exponentially growing monolayer cultures of MCF-7 cells were treated with 20μM nicotine for 120 minutes, followed by a co-exposure to 10μM doxorubicin for 90 minutes. Apoptosis was assayed immediately after exposure to doxorubicin. Twenty micromolar nicotine inhibited doxorubicin-induced apoptosis, based on DNA fragmentation analysis, annexin V binding, and assays for caspase-3 and caspase-8 activities. Treatment with 10μM doxorubicin alone for 90 minutes increased caspase-3 activity to 142% and caspase-8 activity to 126% relative to 100% activity for each enzyme in untreated control cultures. However, a 2 hour pre-treatment with 20μM nicotine reduced the caspase-3 and caspase-8 activities respectively, in cultures treated with doxorubicin to 114% and 101% of control. This corresponds to a 20% inhibition of doxorubicin-induced apoptosis by 20μM nicotine in these cells. In a parallel set of experiments the drug containing medium was replaced with drug free medium after the 90 minute exposure to doxorubicin and then incubated at 37^{o}C for 24 hours. When these cells were examined, inhibition of doxorubicin-induced apoptosis by nicotine could be demonstrated clearly using DNA fragmentation pattern, and annexin V binding, but not on the basis of caspase-3 and caspase-8 activities. Nicotine by itself had no effect on apoptosis, although it inhibited doxorubicin-induced apoptosis. Clonogenicity assays for viability also confirmed the ability of nicotine (10 and 20μM) to protect MCF-7 cells against the cytotoxicity of a 90 minute treatment with doxorubicin (1 and 10μM). Nicotine (10 and 20μM) did not affect the viability of MCF-7/ADR multidrug resistant cells treated with 10μM doxorubicin for 90 minutes. Inhibition of apoptosis in cancer cells by nicotine can diminish the effectiveness of doxorubicin in cancer therapy. Apoptosis is a defense mechanism against carcinogenesis. If nicotine inhibits apoptosis in normal cells or cells progressing towards malignancy, then the risk of secondary malignancies may be higher in patients who use tobacco products during and after doxorubicin treatment.

References: (1) Mortality trends for selected smoking-related cancers and breast cancer. United States 1950-1990. MMWR 1993; 42:857-863. (2) Schnoll RA *et al*. Correlates of tobacco use among smokers and recent quitters diagnosed with cancer. Patient Educ Couns. 2002 Feb;46(2):137-45.

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ACTIVATION OF CALPAIN DURING ß-LAPACHONE-MEDIATED APOPTOSIS IN NQO1-EXPRESSING BREAST CANCER CELLS

Colleen Tagliarino,[1] Jinming Gao,[2] and David A. Boothman[1]

Departments of [1]Radiation Oncology and [2]Biomedical Engineering, Molecular Stress Response Laboratory, Case Western Reserve University, Cleveland, OH 44106-4942

dab30@po.cwru.edu

β-Lapachone (β-Lap), a 1,2-ortho-naphthoquinone, triggers apoptosis in a number of human breast and prostate cancer cell lines through an unique apoptotic pathway that is dependent upon NQO1, a two-electron reductase. Recently, our laboratory showed that β-lap-exposed MCF-7 cells exhibited an early increase in intracellular cytosolic Ca^{2+} from endoplasmic reticulum stores, and that BAPTA-AM (an intracellular Ca^{2+} chelator) blocked these early increases and inhibited all aspects of β-lap-induced apoptosis.

We now show that exposure of NQO1-expressing breast cancer cells to β-lap stimulates an unique proteolytic apoptotic pathway mediated by μ-calpain activation. Upon activation, μ-calpain translocated to the nucleus concomitant with specific nuclear apoptotic-proteolytic events. The apoptotic responses in NQO1-expressing cells to β-lap were significantly delayed and survival enhanced via exogenous expression of calpastatin, a natural inhibitor of μ- and m-calpains. Furthermore, μ-calpain cleaved PARP to a unique fragment (~60 kDa), not previously reported for calpains. We provide evidence that β-lap-induced, μ-calpain-stimulated apoptosis does not involve any known apoptotic caspases; the activated fragments of caspases were not observed after β-lap exposures, nor were there any changes in the pro-enzyme forms as measured by Western blot analyses.

Recent advances in drug delivery have accelerated use of β-lap and its analogs for use against human breast, prostate, as well as lung cancers (all three of these types of tumors have elevated NQO1 levels. The novel drug delivery methods developed, as well as preliminary animal studies, will be discussed. The ability of β-lap to trigger an apparently novel, p53-independent, calpain-mediated apoptotic cell death further support the development of this drug for improved breast cancer therapy. The ability of β-lap and its analogs to augment radiation therapy, including low dose brachytherapy strategies, further supports the development of these types of compounds and delivery methods for the treatment of breast cancer.

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MECHANISTIC COMPARISON OF THE INDUCTION OF CELL DEATH IN ESTROGEN RECEPTOR POSITIVE AND NEGATIVE BREAST CANCER CELLS

Martin Tenniswood, Kerry Gilmore, Louise Flanagan, Caroline Wagner, and Jennifer Hurley

Department of Biological Sciences,
University of Notre Dame, Notre Dame, IN 46556

tenniswood.1@nd.edu

Selective Estrogen Receptor Modulators (SERMs), such as tamoxifen (TAM), have been used for many years as hormonal therapy for advanced breast cancer. TAM is currently being evaluated both for the treatment of early, organ confined breast cancer and for chemoprevention for women at high risk for the disease. In the course of our previous studies, we have shown that tamoxifen exerts its effects on estrogen receptor positive MCF-7 cells by inducing apoptosis in the majority of cells. However, a small proportion of the cells fail to die and acquire an invasive phenotype. This phenomenon is not seen when MCF-7 cells are treated with other agents (such as TNFα, or the pure anti-estrogen ICI 182,780) that also induce apoptosis in these cells, suggesting that TAM induces a unique pattern of gene expression in these cells. Although the primary mechanism of action of TAM is believed to be through the inhibition of estrogen receptor alpha (ERa), the clinical observation that 30% of ER(-) breast cancers respond to TAM treatment suggests that additional non-ERa mediated mechanisms exist. To evaluate the mechanism of TAM induced cell death in ER(+) and ER(-) tumors we have compared the intra-cellular processes leading to the induction of cell death in ER(+) MCF-7 cells and two ER(-) human breast cancer cell lines: SUM-159PT and MDA-MB-435 cells. All three cell lines are growth inhibited by TAM in a time and dose-dependent manner. TAM induces G0/G1 cell cycle arrest and apoptosis in SUM-159PT and MDA-MB-435 cells at levels similar to, or higher than, that observed in the ER(+) MCF-7 cells. Furthermore bax is translocated to the mitochondria and cleaved upon treatment with 10mM TAM after 48 h of treatment, concomitant with release of cytochrome c from the mitochondria into the cytosol. In addition TAM induces PARP cleavage in both ER(-) and ER(+) cell lines suggesting a common apoptotic pathway is induced by TAM. These data suggest that TAM may induce cell death, and possibly an invasive phenotype through mechanisms that are independent of the ER. Further elucidation of the mechanisms of action of TAM in ER(-) breast cancer cells is important for optimizing these agents for the treatment and prevention and treatment of primary tumors and metastatic disease.

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STUDIES OF ENDONUCLEASE G AND ITS POSSIBLE APPLICATION IN INDUCING APOPTOSIS IN CANCER CELLS

Chonglin Yang, Jay Z. Parrish, and Ding Xue

Department of MCD Biology,
University of Colorado, Boulder, CO 80309-0347

xue@spot.colorado.edu

As a normal aspect of animal development and homeostasis, apoptosis plays an essential role in maintaining appropriate cell numbers. Abnormal inactivation of apoptosis can lead to uncontrolled cell growth and the pathogenesis of various human cancers including breast cancer. Apoptosis is controlled and executed by a highly conserved cell death pathway. Recently, a conserved nuclease, endonuclease G, has been identified that plays an important role in fragmenting chromosomal DNA of dying cells to facilitate the cell killing process. EndoG normally localizes to mitochondria in living cells but is released and translocated from mitochondria to nuclei to mediate DNA degradation during apoptosis. Understanding of the molecular mechanisms that regulate the release and activation of EndoG during apoptosis will provide new insights into how apoptosis is executed and new ideas in designing drugs that can be used to specifically induce apoptosis in cancer cells.

We have used a combination of genetic and biochemical approaches and the nematode C. elegans and mammalian cells as experimental systems to identify factors that regulate the release and activation of EndoG in C. elegans and in mammalian cells.

We have set up an in vitro assay for the nuclease activity of EndoG and have used the assay to look for factors that can enhance the nuclease activity of EndoG. So far, we have identified one other nuclease that can synergize with EndoG to degrade DNA in mammalian cells. We are also testing in nematodes the activities of key cell death regulators, such as the CED-9 death inhibitor, in regulating the release of EndoG during apoptosis. We are in the process of screening for mutants in C. elegans that are defective in releasing EndoG during apoptosis and have so far isolated one such mutant. In addition, we have tested some small compounds for activities in releasing EndoG from mitochondria or for activities in activating EndoG in mammalian cells.

These studies of EndoG not only are important in elucidating the mechanisms that regulate apoptotic DNA degradation, a key death execution event, but also may provide new ideas for designing drugs that can be used specifically to induce apoptosis in cancer cells including breast cancer in cancer therapy.

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CRITICAL ROLE OF CASPASE 3 IN MCF-7 BREAST CANCER CELLS IN RESPONSE TO DIFFERENT ANTICANCER AGENTS

XiaoHe Yang,[1] Todd Sladek,[2] Xuesong Liu,[2] Shihe Yang,[2] Brett Ellefson,[2] Janardan Khandekar,[2] Christopher Froelich,[2] and Ann Thor[1]

[1]Department of Pathology, University of Oklahoma Health Science Center; [2]ENH Research Institute, Northwestern University; Department of Microbiology/Immunology, Chicago Medical School

xiaohe-yang@ouhsc.edu

Apoptosis plays important roles in cellular development and homeostasis. Aberrant apoptosis has been associated with the carcinogenesis and therapeutic resistance of cancer. Although apoptosis can be triggered by different stimuli, the involved signaling pathways ultimately converge to activate a group of proteases, called caspases. MCF-7 breast cancer cells, which are resistant to many apoptotic stimuli, have been found to be caspase 3 deficient. To study the correlation between caspase 3 deficiency and therapeutic resistance, we reconstituted caspase 3 in MCF-7 cells and have now characterized their response to several anti-cancer agents, including chemotherapy, radiation and tumor necrosis factor alpha (TNF-a). Caspase 3 was expressed in MCF-7 cells by transfecting the cells with pBabe/puro retroviral vector encoding caspase 3 cDNA. Flow cytometry and MTT assays showed that caspase 3 expression rendered MCF-7 cells significantly more susceptible to these treatments. Apoptosis medicated by the reconstituted caspase 3 was demonstrated by increased DEVD cleavage activities, activation of effector caspases and cleavage of cellular death substrates. Using this caspases 3 specific cell line model, we found that caspase 7 is downstream of caspase 3 and that caspase 6 could be activated by caspase 3 and other apical caspases. Our results also showed that caspase 3 had feedback signals to apical caspases (8, 9 and 2), as demonstrated by both cell-free and whole cells systems. Enhanced mitochondrial depolarization and cytochrome c release was also detected in caspase 3 expressing MCF-7 cells treated with radiation or doxorubicin. These results demonstrated that caspase 3 plays a central role in apoptosis induced by different anti-cancer agents, suggesting that caspase 3 deficiency might contribute to the therapeutic resistance of MCF-7 breast cancer cells.

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DETECTION OF APOPTOSIS IN RAT MAMMARY ADENOCARCINOMAS

Xuexian Zhang and Danuta Malejka-Giganti

Veterans Affairs Medical Center, Research Service (151),
One Veterans Drive, Minneapolis, MN 55417;
Department of Laboratory Medicine and Pathology,
University of Minnesota, Minneapolis, MN 55455

zhang112@tc.umn.edu

Induction of apoptosis is one approach to suppression of cancer. Extensive efforts have focused on induction of apoptosis in cancer cells *in vitro* and elucidation of the mechanisms of apoptosis by antitumor agents. However, data on drug-modulated apoptotic activities in animal tumor models are scarce. Our study examined apoptotic activities in 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced mammary adenocarcinomas in female Sprague-Dawley rats, and potential modulation of apoptosis by treatment of rats with indole-3-carbinol (I3C) at 250 mg/kg or β-naphthoflavone (β-NF) at 20 mg/kg of body weight in ethanol:corn oil (2:3), by oral gavage, three times per week for up to 12 weeks starting 3 weeks after DMBA (one oral dose of 20 mg per 7-week-old rat). Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was performed in tumor sections prepared from paraffin-embedded tissues. The number of apoptotic cells and total number of cells in each field (6 to 8 fields per section) were counted using light microscopy and Image-Plus program. The percentages of apoptotic cells were 2.95 ± 1.23 % (n = 10), 4.93 ± 3.31% (n = 12), and 3.61 ± 1.72 % (n = 11) in tumors from the vehicle-, I3C- and β-NF-treated rats, respectively. Caspase-3 and caspase-9 activities were determined with *N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide and *N*-acetyl-Leu-Glu-His-Asp-*p*-nitroanilide as the specific substrate, respectively, using total tumor protein and colorimetric (absorbance at 405 nm) enzyme–linked immunosorbent assays (ELISA). The relative absorbance for caspase-3 activity was 0.306 ± 0.154 (n = 16) for vehicle-, 0.321 ± 0.158 (n = 20) for I3C- or 0.372 ± 0.136 (n = 15) for β-NF-treated rats. However, the higher activities of caspase-3 in tumors of β-NF- or I3C-treated rats were not significantly different from that of the control group (p = 0.11). Likewise, the relative absorbance for caspase-9 activity of 0.152 ± 0.100 (n = 10) for vehicle-, 0.175 ± 0.075 (n = 8) for I3C- or 0.243 ± 0.105 (n = 8) for β-NF-treated rats was not significantly different among the groups. Protein expression levels of the antiapoptotic gene, Bcl-2, and apoptotic gene Bax, were determined by Western blot analysis using total tumor protein. The pattern of expression of Bcl-2 or Bax was variable, and it did not follow the reported upregulation of Bax by I3C in breast cancer cells *in vitro*. In contrast to inhibition of mammary tumorigenesis by I3C or β-NF administered before DMBA, these compounds used in the post-carcinogen regimen do not significantly change the outcome of tumorigenesis observed in our previous study and apoptotic activities in mammary tumors shown herein.

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ARE DIADENOSINE POLYPHOSPHATES AND/OR FHIT INVOLVED IN ANOIKIS?

Steven M. Frisch

The Burnham Institute

sfrisch@burnham.org

The Fragile Histidine Triad protein FHIT is inactivated by mutation in a large percentage of breast cancer cells. Because the overexpression of FHIT protein induces apoptosis, our project tests the hypothesis that FHIT is a component of the anoikis machinery.

To test this hypothesis, we have applied two approaches. First, we have started a yeast two-hybrid screen for FHIT-interacting proteins, to test the prediction that some of these may be involved in controlling anoikis. Candidate proteins resulting from this screen will be presented.

Secondly, we have been able to reduce FHIT expression in normal mammary epithelial cells (MCF10a) by the use of short-interfering RNA (siRNA) technology. Preliminary results indicate that the effect of this is to protect the cells partially against anoikis. This implicates FHIT function in anoikis. Further results with this and other cell lines will be discussed.

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CELL ADHESION REGULATES FADD LOCALIZATION AND RESPONSE TO FASL

Robert A. Screaton and Steven M. Frisch[1]

The Burnham Institute

sfrisch@burnham.org

Interaction with extracellular matrix can protect cells against several apoptotic stimuli by promoting integrin-initiated cell survival signaling. In principle, cell-matrix contact also might protect cells against death-ligand induced apoptosis by interfering with death receptor signaling complexes. Here, we report that epithelial cell adhesion prevented the FASL-induced formation of a complex between FAS and the adaptor protein FADD, thus preventing efficient caspase-8 activation. As expected, most of the FAS was at the cell surface, yet, surprisingly, most of the FADD in matrix-attached cells was in the nucleus, suggesting that inhibition of FAS signaling was due to sequestration of FADD. In suspended cells, FADD was exported from the nucleus to the cytoplasm, and FAS-FADD complexes formed rapidly in response to FASL. Both FADD nuclear import and export required serine 194 - the known phosphorylation site of FADD - and an intact death effector domain. FADD interacted with the human homologue of yeast msn5 (hmsn5), a nuclear shuttling protein that preferentially transports phosphorylated cargo proteins. A FADD S194A mutant failed to interact with hmsn5, was retained in the cytoplasm and induced apoptosis more potently than wild-type FADD. We propose that the FADD-hmsn5 interaction is necessary but not sufficient for FADD nuclear-cytoplasmic transport and that cell adhesion signals control the response to death ligands by regulating the transport of FADD. The role of cytoskeletal components (actin, microtubules, intermediate filaments) in regulating FADD export is currently being investigated.

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